SUPPLEMENTAL DATA

Nitric oxide synthase forms N-NO-pterin and S-NO-Cys: Implications for activity, allostery, and regulation

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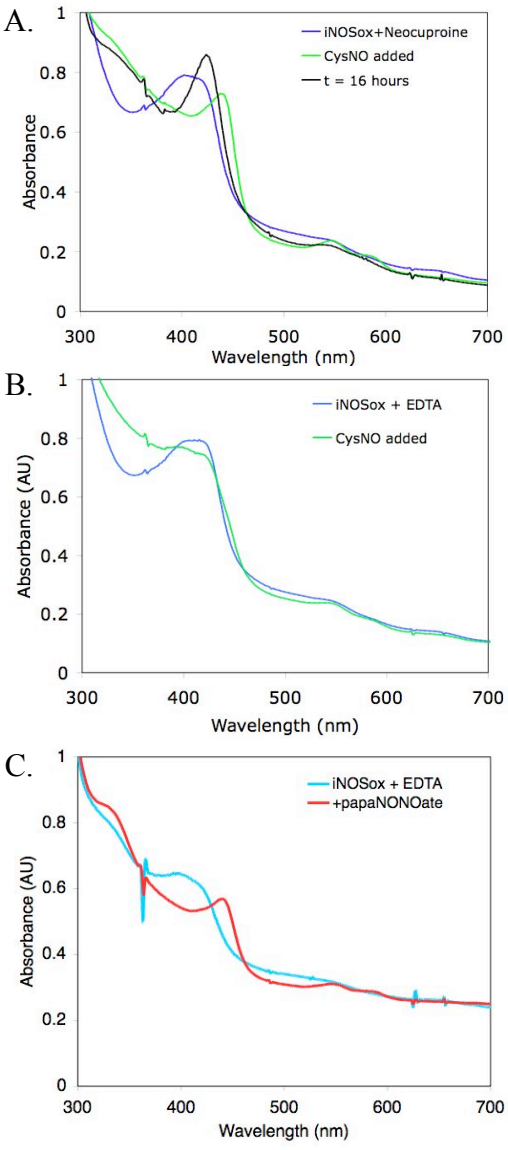

Fig. S1: Nitrosylation of $iNOS_{ox}$ by CysNO and papaNONOate in the presence of metal chelators. *A,* Resting state dimeric $iNOS_{ox}$ (10 μM, blue line) in 40 mM EPPS pH 7.6, 10 μM neocuproine reacts with 500 μM CysNO to form Fe^{III}-NO $iNOS_{ox}$ (green line). Fe^{III}-NO $iNOS_{ox}$ decays slowly to a form a low spin species with ~420 nm absorbance (black line). *B,* Reaction of resting state dimeric $iNOS_{ox}$ (10 μM, blue line) in 40 mM EPPS pH 7.6, 10 μM neocuproine, 0.5 mM EDTA with 500 μM CysNO forms only a small amount of Fe^{III}-NO $iNOS_{ox}$ (green line), suggesting that a divalent metal ion may facilitate Fe^{III}-NO $iNOS_{ox}$ formation. *C,* Reaction of resting state $iNOS_{ox}$ (8 μM, cyan line) in 40 mM EPPS pH 7.6, 0.5 mM EDTA with 400 μM papaNONOate rapidly (< 30 sec) forms Fe^{III}-NO $iNOS_{ox}$ (red line), showing that the $iNOS_{ox}$ heme binds NO in the presence of EDTA.

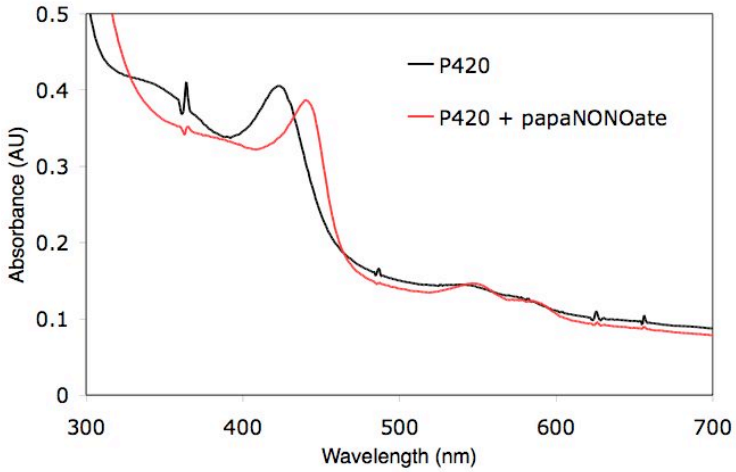

Fig. S2: Nitrosylation of low spin $iNOS_{ox}$ by papaNONOate. Resting state dimeric $iNOS_{ox}$ (5 μM) in 40 mM EPPS pH 7.6 reacted with 250 μM CysNO and decayed to a P420-like species (black line). Addition of 250 μM papaNONOate results in rapid formation of Fe^{III}-NO $iNOS_{ox}$ (red line), indicating that this P420–like species does not have NO bound on the heme iron.

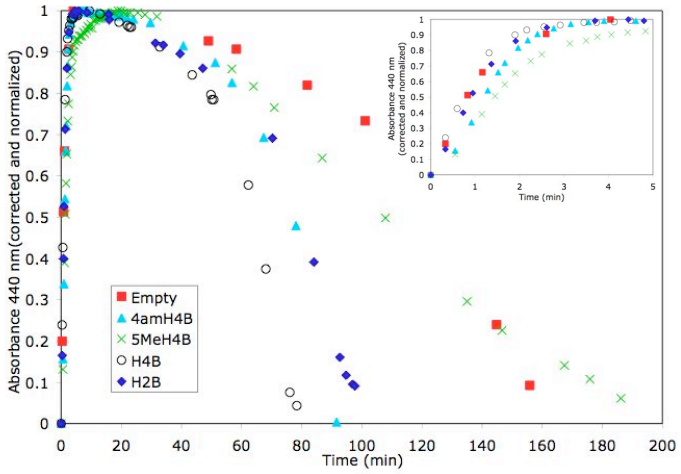

Fig S3. Pterin impacts on $iNOS_{ox}$ nitrosation reactions: $iNOS_{ox}$ with H_4B, H_4B analogues (4-amino-H_4B, H_2B, or 5-methyl-H_4B), or with no pterin bound reacted rapidly with CysNO to form Fe^{III}-NO, achieving complete conversion to Fe^{III}-NO between 3-5 minutes (inset). Fe^{III}-NO $iNOS_{ox}$ decayed to a species with a ~420 nm Soret. Fe^{III}-NO $iNOS_{ox}$ with H_2B, H_4B, or 4-amino-H_4B bound decayed more quickly (t = 70 – 100 min), than did Fe^{III}-NO $iNOS_{ox}$ with 5-methyl-H_4B or no pterin bound (t = 160 – 190 min).

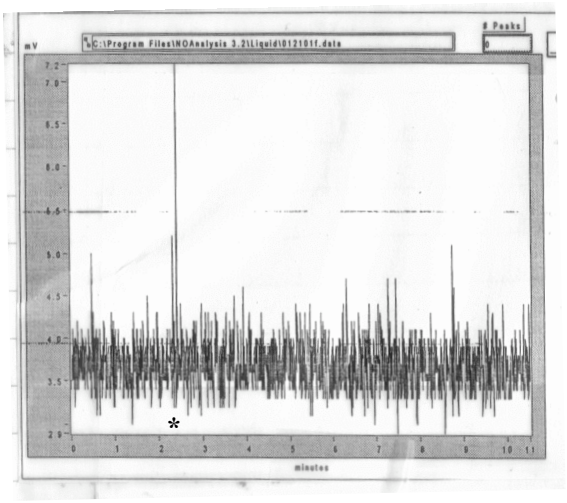

Fig. S4: Chemiluminescence signal from a control sample containing iNOS reaction mixture without NADPH (full-length iNOS, FAD/FMN, L-Arginine, H_4B) and 5 mM nitrite following micro-dialysis. Injection peak is marked (*).

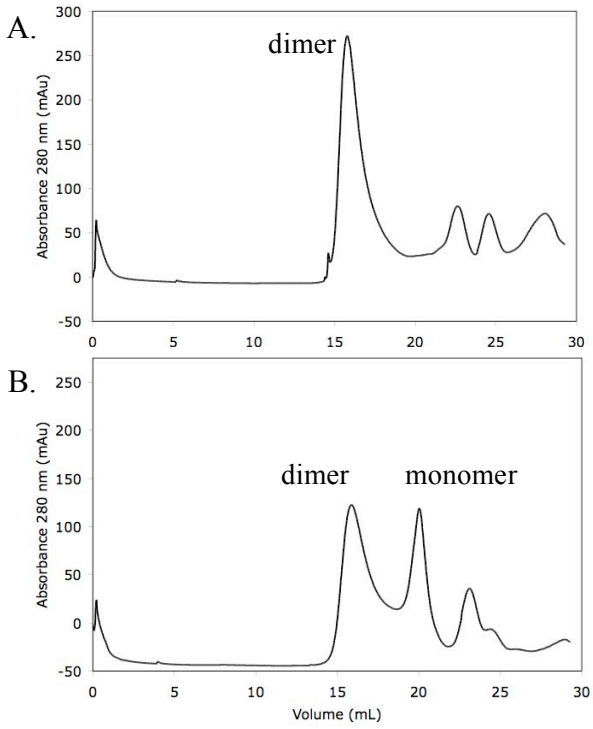

Fig. S5: Nitrosation promotes monomerization of $iNOS_{ox}$. ***A,*** Size exclusion chromatography shows that $iNOS_{ox}$ (160 μM heme, $+H_4B$, +L-Arginine) is predominantly dimeric. ***B,*** $iNOS_{ox}$ (160 μM heme, $+H_4B$, +L-Arginine) nitrosated with GSNO (10 mM) for 15 min elutes as a mixture of monomer (~40%) and dimer (~60%). Under these conditions the elution volumes for dimeric and monomeric $iNOS_{ox}$ are 15.8 mL and 20.0 mL, respectively.

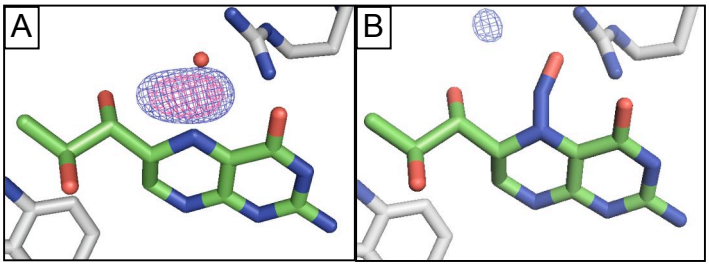

Fig. S6: ***A****,* A water molecule does not account for the observed electron density: strong residual Fo-Fc electron density occurs between the modeled water molecule and pterin-N5. This Fo-Fc map was calculated from a model with water hydrogen-bonded to unmodified pterin, and contoured at 3σ (blue) and 5σ (magenta). The density supports a diatomic covalent modification of the pterin. ***B****,* N-NO modified pterin accounts for the observed electron density: no significant residual Fo-Fc electron density occurs near the N-NO-pterin, with map contoured at 3σ (blue).

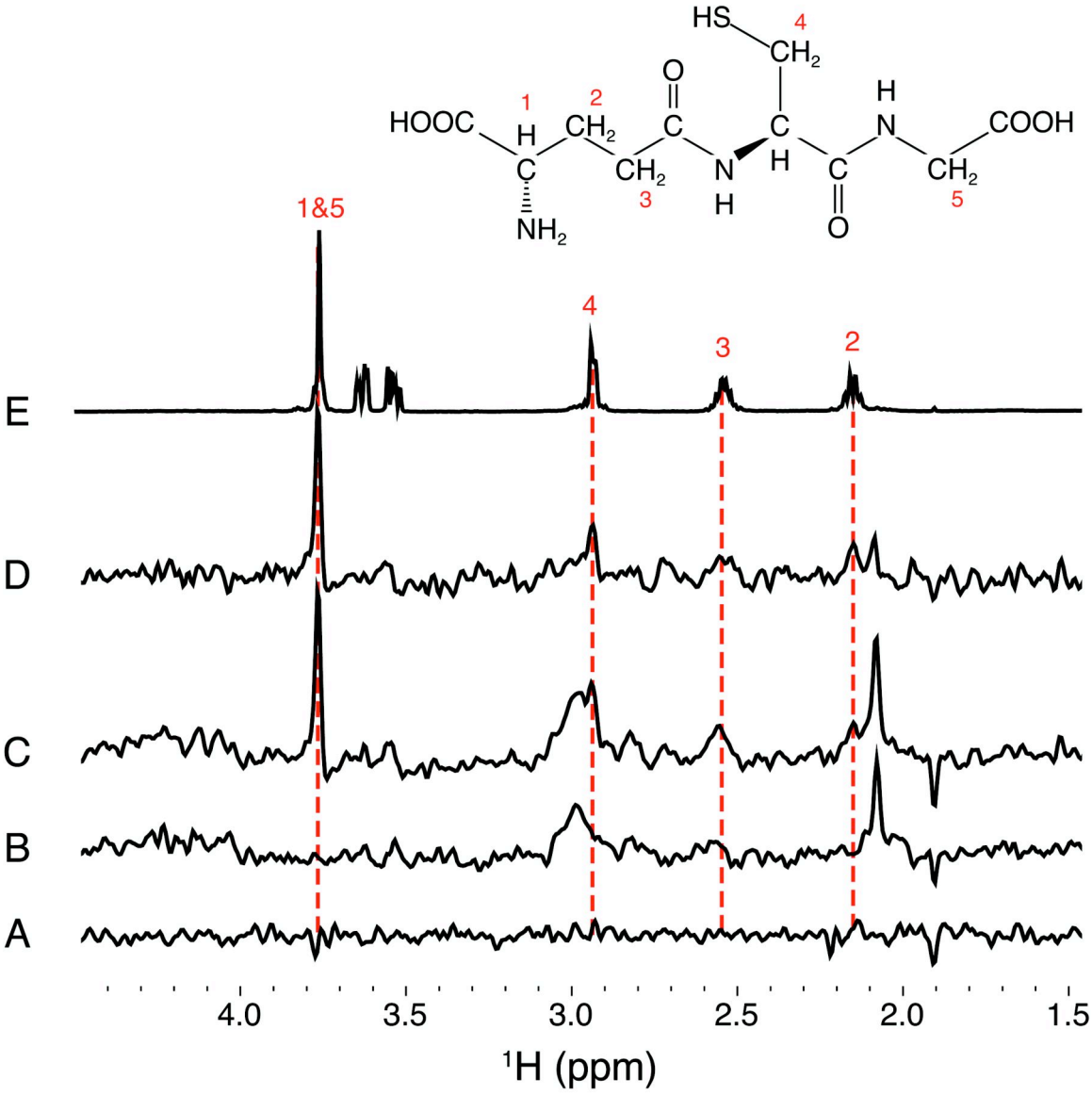

Fig. S7: ^{1}H STD-NMR spectra reveal interactions of GSH with $iNOS_{ox}$. ***A,*** GSH alone in solution does not produce significant STD signals under the experimental conditions. ***B,*** STD spectrum of $iNOS_{ox}$ in NMR buffer without GSH. The small peaks observed are attributed to residual glycerol (3.5 and 3.6 ppm) and EPPS (3.0 and 2.1 ppm). ***C,*** STD spectrum of $iNOS_{ox}$:GSH shows a large GSH proton signal at 3.75 ppm as well as smaller peaks attributable to additional GSH protons and protons present in the protein only sample. ***D,*** The STD signals associated with the interactions of GSH with $iNOS_{ox}$ are revealed when the STD spectrum of $iNOS_{ox}$ is subtracted from the STD spectrum of $iNOS_{ox}$:GSH. The predominant resonance at 3.75 ppm and smaller resonances at 2.9, 2.55 and 2.15 ppm are assignable to GSH protons (red dashed lines). ***E***, 1D ^{1}H NMR spectrum of GSH (1mM) and $iNOS_{ox}$ (32 μM heme) reveals GSH resonances (3.75, 2.9, 2.55, 2.15 ppm) and residual glycerol resonances (3.5-3.65 ppm) in the NMR sample. Identical STD experiments were collected for all samples and are described in the experimental methods section

Sample	Peptide	S-Nitrosylated residue(s)
$^{14}N,^{16}O$-CysNO-treated $iNOS_{ox}$	TC(NO)KSC(NO)LGSIM(ox)NPK	Cys104, Cys109
	KSKSC(NO)LGSIMNP	Cys109
	ATSDFTC(NO)KS	Cys104
	ATSDFTC(NO)KSK	Cys104
$^{15}N,^{18}O$-CysNO-treated $iNOS_{ox}$	SKSC(NO)LGSIMNPK	Cys109
	SC(NO)LGSIMNPK	Cys109
	SC(NO)LGSIM(ox)NPK	Cys109
	KSKSC(NO)LGSIMNPK	Cys109
	KSC(NO)LGSIMNPK	Cys109
	DFTC(NO)KSKSC(MMTS)LGSIMNPKSLTR	Cys104

Table S1: Summary of S-nitrosylated $iNOS_{ox}$ peptides identified by using tandem mass spectrometry on protein samples treated with either $^{14}N^{16}O$-CysNO or isotopically labeled $^{15}N^{18}O$-CysNO. The samples were treated with methyl methanethiosulfonate (MMTS) to protect free thiols and digested with elastase, trypsin and chymotrypsin. The peptides were analyzed using multidimensional protein identification technology on an LCQ ion trap mass spectrometer. The resulting tandem mass spectra were interpreted and modified residues identified using SEQUEST. C(NO) indicates S-nitrosylated Cysteine, C(MMTS) indicates MMTS modification, and M(ox) indicates oxidized methionine.